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14. ABSTRACT

The original project for the grant was to determine if an observed modification of EAF1 and EAF2 altered how EAF2 is regulated in the normal prostate and in prostate cancer. However, co-immunoprecipitation results showed the modification was a covalent dimerization. The project then shifted to determining how the tumor suppressor ELL-associated factor 2 (EAF2) interacts Fork-head box protein A1 and what the ramifications of that interaction would be. Work on specific aim 1; determine how FOXA1 interacts with EAF2, revealed over-expressed FOXA1 binds to EAF2, EAF2 binds to the N-terminus and C-terminus of FOXA1, and over-expression of FOXA1 and EAF2 reduces FOXA1 and EAF2 protein levels, but it remains unknown if over-expression of FOXA1 and EAF2 affects FOXA1 and EAF2 protein stability. Work on specific aim 2; determine the effect of EAF2 on FOXA1-mediated transcription and on FOXA1-mediated cell growth and survival, revealed that loss of EAF2 reduces PSA protein and mRNA levels and increases FOXA1 protein levels, over-expression of FOXA1 reduces PSA-promoter activity, and over-expression of FOXA1 and EAF2 results in an intermediary level of cell survival and proliferation, compared to FOXA1 alone which promoted cell growth and survival and EAF2 alone which promoted cell death. This project led to a dissertation and a PhD.

15. SUBJECT TERMS none provided

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Introduction

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer death in men in the US (*1*). Prostate cancer results from the accumulation of mutations that affect regulatory pathways controlling cell growth, differentiation, and death. ELL-associated factor 2, also known as androgen up-regulated 19, (EAF2/U19) is a putative transcription factor and was discovered to be a protein up-regulated by the androgen receptor in the normal prostate. Over-expression of EAF2 induces apoptosis in prostate cancer cell lines while EAF2 is down-regulated in the majority of human prostate cancer specimens (*2*). Furthermore, loss of EAF2 in mice results in prostatic intraepithelial neoplasia, the putative precursor of prostate cancer (*3*). ELL-associated factor 1 (EAF1) is the only homolog of EAF2 in mammalians and is believed to play a role in leukemia (*4, 5*). Studying how the EAF family proteins are regulated and which proteins EAF2 interacts with and whether that interaction plays a role in prostate cancer development and progression may provide methods to treat prostate cancer.

The initial project was based on preliminary data which showed the presence of modified EAF1 and EAF2, which are about two times heavier than the unmodified EAF1 and EAF2. Furthermore, preliminary data showed that the addition of MG132 increases EAF1 and EAF2 protein levels and causes EAF2 to localize to the nucleus. An *in silico* analysis of EAF1 and EAF2 amino acid sequences using SUMOplot (*6*) predicted 2 potential sumoylation sites in EAF1, K54 and K174, as well as two potential sumoylation sites in EAF2, K18 and K164. Increased sumoylation caused a drop in EAF1 levels and a shift in EAF1 localization. The three specific aims for the project were as follows: specific aim 1 was to determine the nature of the modification of EAF1 and EAF2, specific aim 2 was to determine if the modification affects EAF1 and EAF2 function, and specific aim 3 was to understand the roles of sumoylation and protein degradation in EAF1 and EAF2 localization. However, due to the difficulties of providing conclusive evidence that the observed EAF1 and EAF2 modifications were a covalent dimerization, a new project was devised.

The preliminary evidence for the new project was an RNAi phenotypic enhancer screen using the *C. elegans* in which the homolog of EAF2, *eaf-1*, had been knocked out. Loss of *eaf-1* results in reduced fertility and a positive hit would be the loss of a second gene that renders the *C. elegans* sterile. There was a brief focus on two of the positive hits from the screen, *hmg-1.2*, the *C. elegans* homolog to HMGB1 and *pha-4*, the *C. elegans* homolog to FOXA1. HMGB1 is high mobility group box 1 protein, a non-histone non-specific DNA binding protein in the nucleus, a mediator of autophagy in the cytoplasm, and a cytokine when it is extracellular (*7*). Furthermore, high levels of HMGB1 are associated with worse outcomes in prostate cancer (*8*). FOXA1 is forkhead box protein A1 and it is a pioneer factor for the androgen receptor (AR). FOXA1 binds to chromatin, rearranges the nucleosomes to make open chromatin, and then AR binds to FOXA1 to begin gene transcription (*9, 10*). FOXA1 is required for the development of ductal epithelial cells in the prostate and high levels of FOXA1 in advanced prostate cancer is indicative of a poor patient outcome (*11, 12*). There was also preliminary evidence that FOXA1 and EAF2 associate while HMGB1 and EAF2 did not associate. EAF2 was observed to alter HMGB1 localization, but this was only in ~30% of co-transfected cells. Therefore the decision was made to focus on the interaction of FOXA1 and EAF2. The three specific aims for the new project were as follows: specific aim 1 will determine how FOXA1 interacts with EAF2 and specific aim 2 will determine the effect of EAF2 on FOXA1-mediated transcription and on FOXA1-mediated cell growth and survival. The new set of specific aims were completed and turned into a Ph.D. dissertation.

Body

EAF1 and EAF2 form homodimers when over-expressed

In 2011, when the original grant was submitted, it was thought that the observed modification (Figure 1) was likely due to either polyubiquitination or polysumoylation. Due to concerns over the size of the modification, a co-immunoprecipitation was performed to eliminate the possibility of a dimerization. As seen in Figure 2, the co-immunoprecipitation showed that the post-translational modification was due to covalent dimerization. At the time it was shown that the modification was due to dimerization, EAF1 mutants were generated that lack the

potential sumoylation sites by mutating the lysine residues (K) that were predicted to be sumoylated to arginine residues (R). A co-immunoprecipitation was performed to determine if the EAF1 K54R or the EAF1 K174R mutant showed altered dimerization. As can be seen in Figure 3, the mutants underwent dimerization in a manner similar to the wild-type protein, but the protein levels of the mutated EAF1 were reduced.

Loss of hmg-1.2 *or* pha-4 *in* eaf-1 *knockout* C. elegans *results in sterility*

Based on this evidence, the decision was made to switch projects. At the same time, a screen was performed in *C. elegans* to find genes that may interact with EAF2. In *C. elegans*, there is a homolog to EAF1 and EAF2, *eaf-1*. When *eaf-1* is knocked out in *C. elegans*, reduced fertility, shortened body length, and increased death is observed. The screen was designed to find phenotypic enhancers, mutations that make a phenotype worse (*13, 14*). The read-out for the screen was reduced fertility, with a positive RNAi resulting in severely reduced fertility or sterility. Two of the hits from the screen, which used a library of nuclear factors, were RNAi knockdowns of *hmg-1.2*, the *C. elegans* homolog of HMGB1, and *pha-4*, the *C. elegans* homolog of FOXA1. Figure 4 shows that loss of either *hmg-1.2* or *pha-4* in *eaf-1* knockout *C. elegans* results in sterility. After examining the resulting offspring (Figure 5), it was noted that the *eaf-1* knockout *C. elegans* treated with *hmg-1.2* RNAi were prone to vulval extrusion, where the guts of the *C. elegans* come out through the vulva due to cuticle abnormalities, the *eaf-1* knockout *C. elegans* treated with *pha-4* RNAi suffered egg degeneration, and there were no notable problems in the other *C. elegans* samples.

In addition to the results of the *C. elegans* screen, *hmg-1.2* and *pha-4* had mammalian homologs that were known to play a role in prostate cancer. One of the mammalian homologs of *hmg-1.2* is HMGB1, which is known to play a role in autophagy and it has been observed that high levels of HMGB1 indicates a poor outcome in prostate cancer (*7, 8*). One of the mammalian homologs of *pha-4* is FOXA1, which is a pioneer factor for the androgen receptor, and it has been observed that FOXA1 levels are elevated in castration-resistant prostate cancer and high FOXA1 protein levels indicate a poor outcome in prostate cancer (*12, 15*).

FOXA1 associates with EAF2; Co-transfection of HMGB1 and EAF2 causes HMGB1 to translocate to the nucleus.

Several experiments were performed in order to determine whether to focus on HMGB1 or FOXA1. Co-transfections of *hmg-1.2* and *eaf-1* and HMGB1 and EAF2 were performed in 293 cells (Figure 6). The co-transfections showed that the presence of EAF2 family proteins caused HMGB1 family proteins to translocate to the cytoplasm. The HMGB1 family proteins are DNA-binding proteins in the nucleus, mediators of autophagy in the cytoplasm, and cytokines when extra-cellular (*7*). Co-transfections of FOXA1 and EAF2 showed that EAF2 associated with FOXA1 (Figure 7) and both EAF2 and FOXA1 protein levels were reduced when co-transfected compared to transfection with the empty vector (Figure 8). Based on the results and the literature on FOXA1, the decision was made to focus on the interaction of FOXA1 and EAF2.

FOXA1 and EAF2 interaction hypothesis

The hypothesis for the new project was based on the known roles of FOXA1 and EAF2 in prostate cancer. FOXA1 is known to promote tumor progression in metastatic and castration-resistant prostate cancer, while EAF2 is known to be a tumor suppressor. This suggested the model that when EAF2 levels are reduced during prostate cancer, FOXA1 levels rise, shifting the cell into a more oncogenic state (Figure 9). The hypothesis was that EAF2 associates with FOXA1 to modulate AR transactivation.

FOXA1 associates with the N-terminus and the C-terminus of EAF2

The next set of experiment was performed in order to determine which regions of EAF2 are required for the association of FOXA1 and EAF2. As seen in Figures 10 and Figure 11, FOXA1 associated with both the N-terminus and C-terminus of EAF2. Both the N-terminus and C-terminus of EAF2 are involved in transcription elongation (*5*, *16*).

Over-expression of FOXA1 and EAF2 reduce the protein levels of both proteins; FOXA1 protein levels increase and PSA protein and mRNA levels decrease when EAF2 is reduced

One of the next questions was whether the observed reduction of FOXA1 and EAF2 protein levels when co-expressed occurred at the endogenous level. FOXA1 protein levels were measured in the anterior prostates of EAF2 knockout mice. As seen in Figure 12, FOXA1 protein levels were elevated in EAF2 knockout mice. When EAF2 is knocked down in LNCaP cells, FOXA1 protein levels increase and PSA protein levels decrease, which suggests that loss of EAF2 alters FOXA1-mediated AR signaling (Figure 13). In addition, PSA mRNA levels are decreased in EAF2 knockdown LNCaP cells (Figure 14).

Over-expression of FOXA1 inhibits PSA promoter activity

The next set of experiments was based on the discovery that loss of EAF2 reduced PSA mRNA levels. First, a luciferase assay was performed to measure PSA promoter activity when FOXA1 is over-expressed in C4-2 cells. The luciferase assay showed that FOXA1 reduced PSA promoter activity in a dose-dependent manner in the presence and absence of supplemental androgen, which matched previous reports (Figure 15) (*17*). This supported the earlier observed result that loss of EAF2 increased FOXA1 and decreased PSA.

Over-expression of FOXA1 and EAF2 produces an intermediate cell survival and proliferation phenotype

The final set of experiments that were performed was colony formation assays to determine if co-transfection of FOXA1 and EAF2 in LNCaP cells would alter cell growth and survival. It was hypothesized that FOXA1 would increase colony formation, EAF2 would decrease colony formation and FOXA1+EAF2 would result in an intermediate phenotype. The results from the colony formation assays (Figure 16) matched the hypothesis.

Results Conclusion

The plan for the FOXA1 and EAF2 project was to spend months 1-14 determining how FOXA1 and EAF2 interact by confirming that FOXA1 and EAF2 associate, determining the regions of EAF2 required for EAF2 and FOXA1 to associate, and determining if co-expression of FOXA1 and EAF2 altered the stability of FOXA1 and EAF2 proteins. Months 14-24 were to be used to determine if EAF2 regulates FOXA1-mediated androgen

receptor signaling and if co-expression of FOXA1 and EAF2 regulated cell growth and survival in a prostate cancer cell line. The plan was closely adhered to and should yield a paper in the near future.

Key Research Accomplishments

- EAF1 and EAF2 each form covalent dimers.
- The *C. elegans* homologs of HMGB1 and EAF2, *hmg-1.2*and *eaf-1,* interact and loss of *hmg-1.2* in *eaf-1* knockout *C. elegans* results in sterility.
- The *C. elegans* homologs of FOXA1 and EAF2, *pha-4* and *eaf-1*, interact and loss of *pha-4* in *eaf-1* knockout *C. elegans* results in sterility due to egg degenerations.
- Over-expression of HMGB1 and EAF2 causes HMGB1 to translocate from the nucleus to the cytoplasm.
- Transfected FOXA1 and EAF2 associate with cells from a prostate cancer cell line.
- FOXA1 associates with the N-terminus and C-terminus of EAF2.
- Co-transfection of FOXA1 and EAF2 reduces FOXA1 and EAF2 protein levels compared to when FOXA1 and EAF2 are co-transfected with an empty vector.
- FOXA1 protein levels are elevated in the prostate tissue of EAF2 knockout mice.
- Knockdown of EAF2 in a prostate cancer cell line increases FOXA1 protein levels and decreases PSA protein and mRNA levels.
- Over-expression of FOXA1 reduces PSA promoter activity.
- Over-expression of FOXA1 increases colony formation, over-expression of EAF2 decreases colony formation, and over-expression of FOXA1 and EAF2 results in an intermediate phenotype.

Reportable Outcomes

- Submitted an abstract and presented a poster at the November 2011 Society for Basic Urology Research Conference entitled “Enhancement of the eaf-1KO phenotype by hmg-1.2” (*18*)

- Submitted an abstract and presented a poster at the October 2012 Great Lakes Nuclear Receptor Conference entitled "Exploring mechanisms of EAF2 action – from RNAi screen in *C. elegans* to mammalian analysis" (*19*)
- Submitted and successfully defended a doctoral dissertation entitled "EAF2 Associates with FOXA1 and EAF2 Alleviates FOXA1-Mediated Repression of Androgen Receptor Transactivation" in December 2013 (*20*)
- Ph.D. degree will be awarded by the end of April 2014

Conclusion

The project was designed to investigate how the tumor suppressor EAF2 is regulated in the normal prostate and dysregulated during prostate cancer and to support the doctoral training of the primary investigator. Initially, the focus of the research was to determine the role a protein modification of EAF2 played in the regulation of EAF2, but the focus shifted to investigating a potential interaction between EAF2 and FOXA1 and the role the interaction played regulating EAF2 and FOXA1, FOXA1-mediated androgen receptor signaling, and cell growth and survival. This research did show that the interaction between FOXA1 and EAF2 may represent a feedback loop that modulates androgen receptor signaling (Figure 17) and may play a role in regulating cell growth and survival. The primary investigator is also working on submitting a paper based on this research. In addition, the primary investigator has successfully submitted a dissertation based on the research this grant supported and will officially graduate with a PhD in April 2014. The project has successfully found an interesting mechanism by which EAF2 can modulate androgen receptor signaling and further elucidates the effects of EAF2 down-regulation in prostate cancer.

References

1. R. Siegel, D. Naishadham, A. Jemal, *CA Cancer J Clin* **62**, 10 (Jan-Feb, 2012).
2. W. Xiao *et al.*, *Cancer Res* **63**, 4698 (Aug 1, 2003).
3. W. Xiao *et al.*, *Oncogene* **27**, 1536 (Mar 6, 2008).
4. F. Simone *et al.*, *Blood* **98**, 201 (Jul 1, 2001).
5. F. Simone, R. T. Luo, P. E. Polak, J. J. Kaberlein, M. J. Thirman, *Blood* **101**, 2355 (Mar 15, 2003).
6. Abgent. (Abgent, 2010).
7. D. Tang, R. Kang, H. J. Zeh, 3rd, M. T. Lotze, *Biochim Biophys Acta* **1799**, 131 (Jan-Feb, 2010).

8. H. Ishiguro *et al.*, *Prostate* **64**, 92 (Jun 15, 2005).
9. J. H. Taube, K. Allton, S. A. Duncan, L. Shen, M. C. Barton, *J Biol Chem* **285**, 16135 (May 21, 2010).
10. M. A. Augello, T. E. Hickey, K. E. Knudsen, *The EMBO journal* **30**, 3885 (Oct 5, 2011).
11. N. Gao *et al.*, *Development* **132**, 3431 (Aug, 2005).
12. J. Gerhardt *et al.*, *Am J Pathol* **180**, 848 (Feb, 2012).
13. J. M. Perez-Perez, H. Candela, J. L. Micol, *Trends in genetics : TIG* **25**, 368 (Aug, 2009).
14. D. Fay, *WormBook : the online review of C. elegans biology*, 1 (2006).
15. N. Gao *et al.*, *Molecular endocrinology (Baltimore, Md* **17**, 1484 (Aug, 2003).
16. K. Saso, T. Ito, S. Natori, K. Sekimizu, *J Biochem* **133**, 493 (Apr, 2003).
17. H. J. Lee, M. Hwang, S. Chattopadhyay, H. S. Choi, K. Lee, *Biochem Biophys Res Commun* **367**, 481 (Mar 7, 2008).
18. A. Lipton, in *Society For Basic Urologic Research Fall 2011 Symposium*. (Las Vegas, NV, 2011).
19. A. L. Keener, in *Great Lakes Nuclear Receptor Conference*. (Chicago, IL, 2012), vol. 1, pp. 89.
20. A. L. Keener, University of Pittsburgh (2014).

Appendix A: Figures

Figure 1

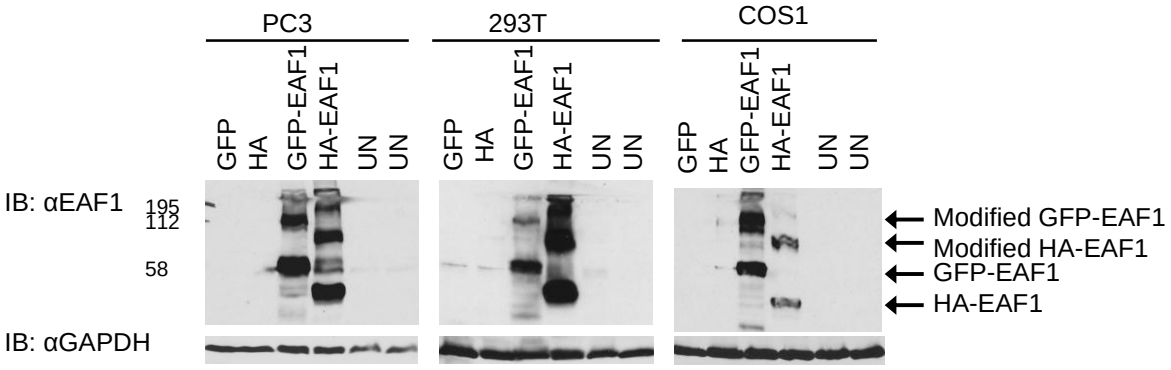

Figure 1: Modified EAF1 is observed in multiple cell lines. PC3, 293T, and COS1 cells were transfected with GFP-tagged EAF1 (GFP-EAF1), HA-tagged EAF1 (HA-EAF1), GFP empty vector (GFP), or HA empty vector (HA). Lysates were probed using anti-EAF1 antibody (top western blots) or with anti-GAPDH antibody (bottom western blots).

Figure 2

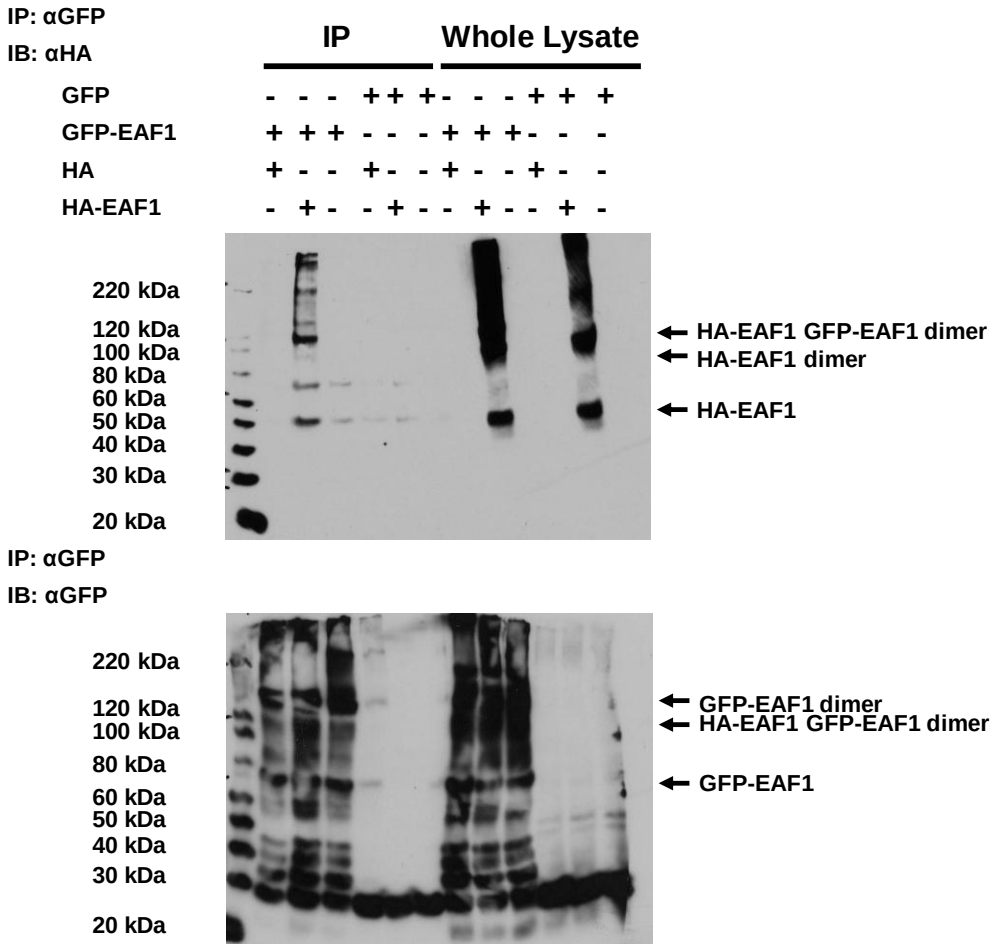

Figure 2: EAF1 forms a homodimer. GFP-tagged EAF2 (GFP-EAF2) and the GFP-tagged vector (GFP) were co-transfected with HA-tagged EAF2 (HA-EAF2), the HA-tagged empty vector (HA) or were transfected alone into 293 cells. Cell lysates were immunoprecipitated (IP) using anti-GFP antibody-conjugated agarose beads, and blots of the IP lysates and whole cell lysates (whole lysates) were probed using anti-HA antibody (top image)and anti-GFP antibody (bottom image).

Figure 3

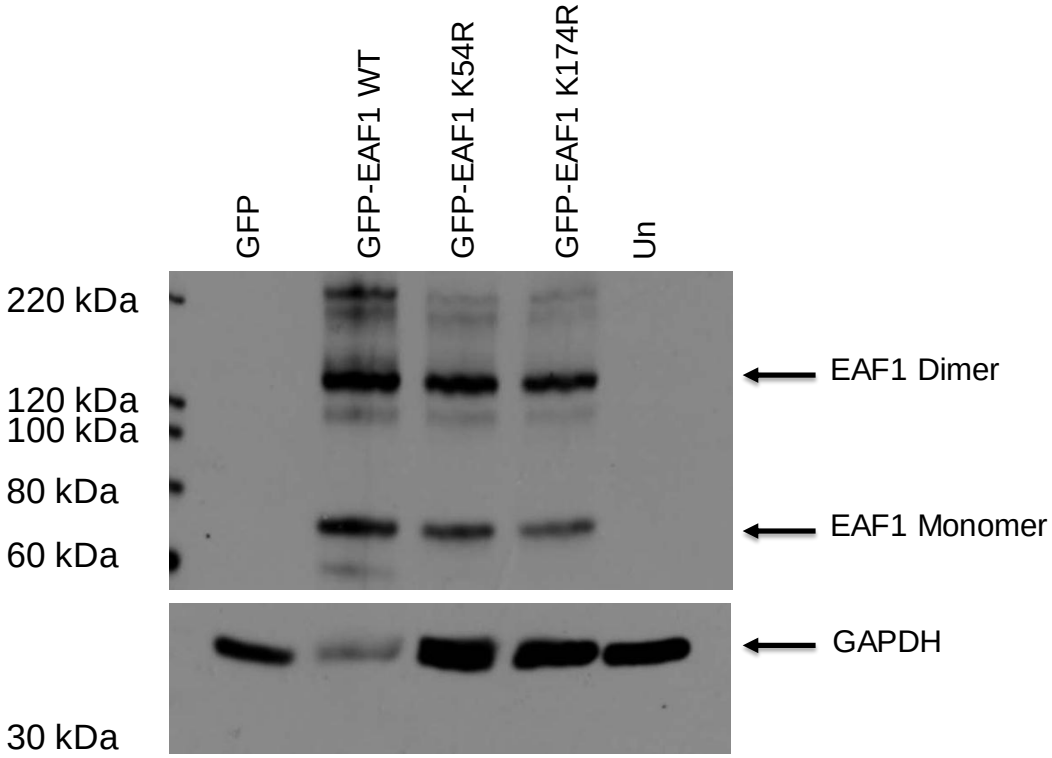

Figure 3: Mutating the potential sumoylation sites did not inhibit dimerization but did reduce protein levels. COS1 cells were transfected with wild-type GFP-EAF1 (GFP-EAF1 WT), GFP-EAF1 K54R, GFP-EAF1 K174R or with the GFP empty vector (GFP). The blots were probed with goat anti-EAF1 antibody (upper blot) and goat anti-GAPDH (lower blot).

Figure 4

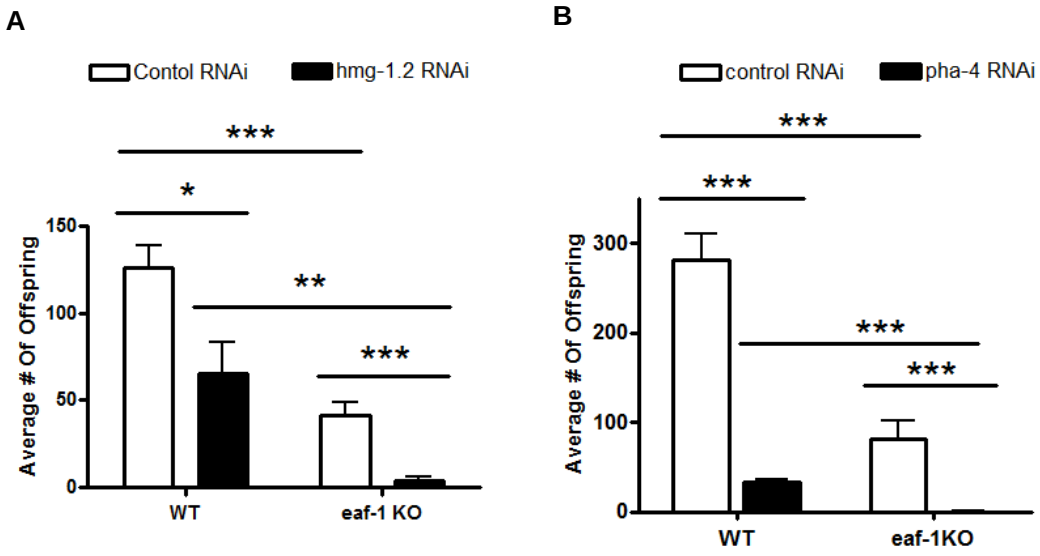

Figure 4: Loss of either *hmg-1.2* or *pha-4* in *eaf-1* knockout *C. elegans* results in sterility. A) Wild-type *C. elegans* worms (WT) or *eaf-1* knockout worms (eaf-1KO) were placed on RNAi agarose plates treated with IPTG and inoculated with *E. coli* that had been transformed with either control RNAi or *hmg-1.2* RNAi. Each experiment was performed with 6 sample replicates. Columns represent the average number of offspring for 7 separate experiments. Significance was determined by student's *t*-test. B) Wild-type worms or *eaf-1* knockout worms were placed on RNAi agarose plates treated with β-galactose and inoculated with *E. coli* that had been transformed with either control RNAi or *pha-4* RNAi. Significance was determined by student's *t*-test. *=$p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$.

Figure 5

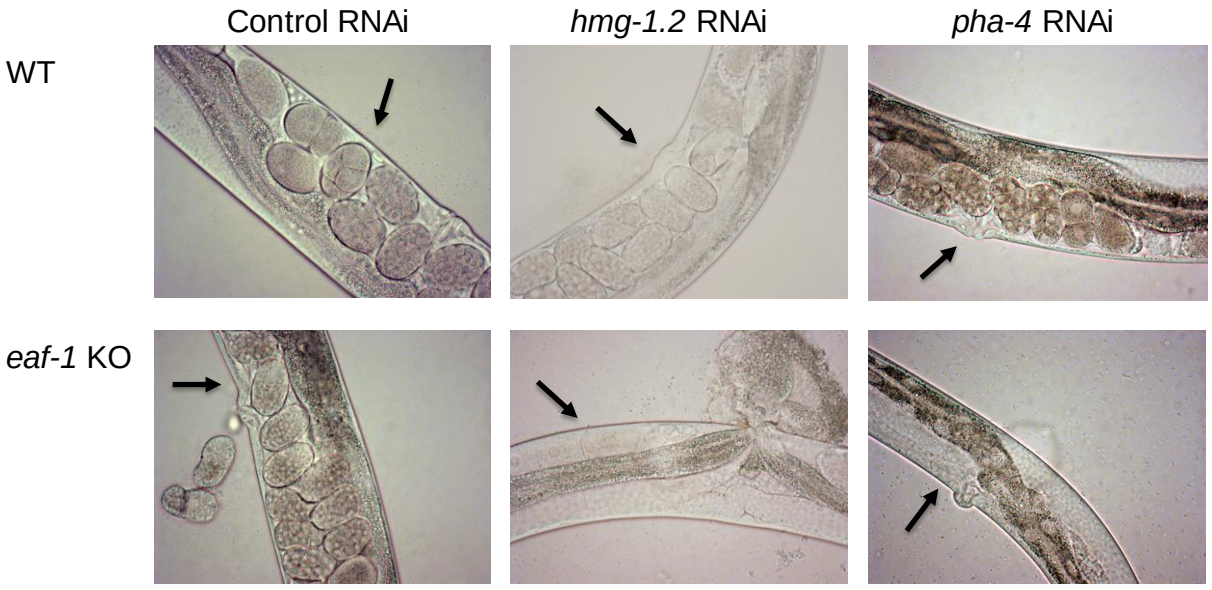

Figure 5: Loss of either *hmg-1.2* or *pha-4* in *eaf-1* knockout *C. elegans* results in abnormal egg development. Representative images showing egg development in adult wild-type (WT) and *eaf-1* KO (*eaf-1* KO) *C. elegans* that have been treated with control, *hmg-1.2*, or *pha-4* RNAi. The arrows indicate the gonads in which egg development occurs. Extrusion of the guts through the vulva was frequently observed the *eaf-1* knockout *C. elegans* treated with *hmg-1.2* RNAi, possibly disrupting egg development and creating a situation where the eggs hatch inside the body of their parent. Egg development is absent in the *eaf-1* KO *C. elegans* treated with *pha-4* RNAi. The arrows indicate the gonads in which the eggs typically develop.

Figure 6

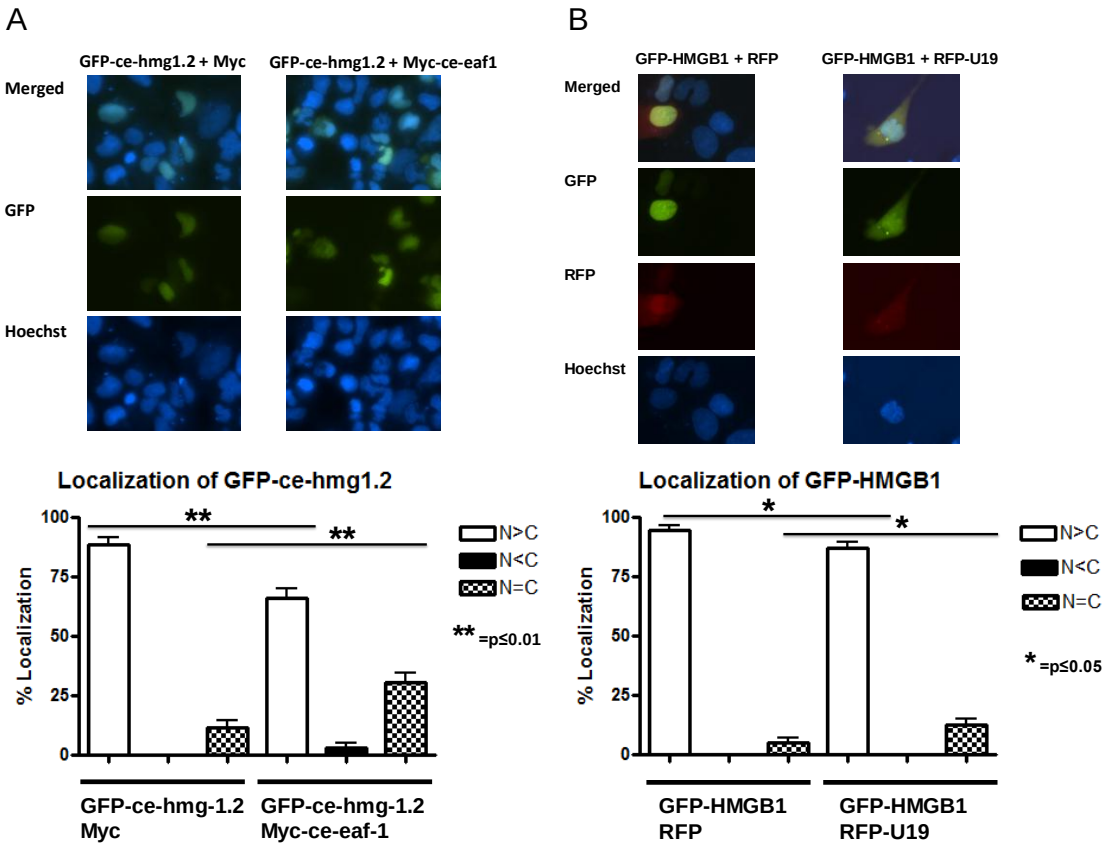

Figure 6: Co-transfection of EAF family proteins with HMGB1 family proteins causes HMGB1 family protein to move to the cytoplasm. A) 293 cells were transfected with GFP-tagged hmg-1.2 (GFP-ce-hmg-1.2) and either Myc-tagged empty vector (Myc) or Myc-tagged eaf-1 (Myc-ce-eaf1). Cells were stained with 0.001% Hoechst 33342 solution 18 hours post transfection. Significance determined by Student's *t*-test. B) 293 cells were transfected with GFP-tagged HMGB1 (GFP-HMGB1) and either RFP-tagged empty vector (RFP) or RFP-tagged EAF2/U19(RFP-U19). Cells were stained with 0.001% Hoechst 33342 solution 18 hours post transfection. Significance determined by Student's *t*-test.

Figure 7

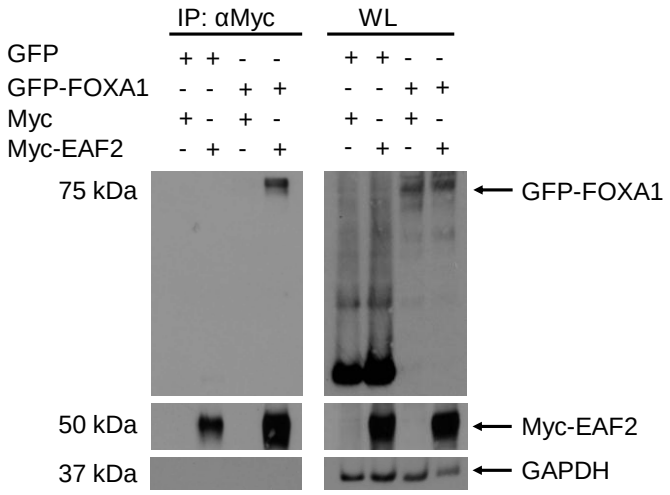

Figure 7: FOXA1 and EAF2 associate when overexpressed in a prostate cancer cell line. GFP-tagged FOXA1 (GFP-FOXA1) and the GFP-tagged vector (GFP) were co-transfected with either Myc-tagged EAF2 (Myc-EAF2) or the Myc-tagged empty vector (Myc) into PC3 cells. Cell lysates were immunoprecipitated (IP) using anti-Myc antibody-conjugated agarose beads, and blots of the IP lysates and whole cell lysates (WL) were probed using anti-GFP antibody (top images), anti-Myc antibody (middle images), and anti-GAPDH antibody (bottom images). Four replications were performed.

Figure 8

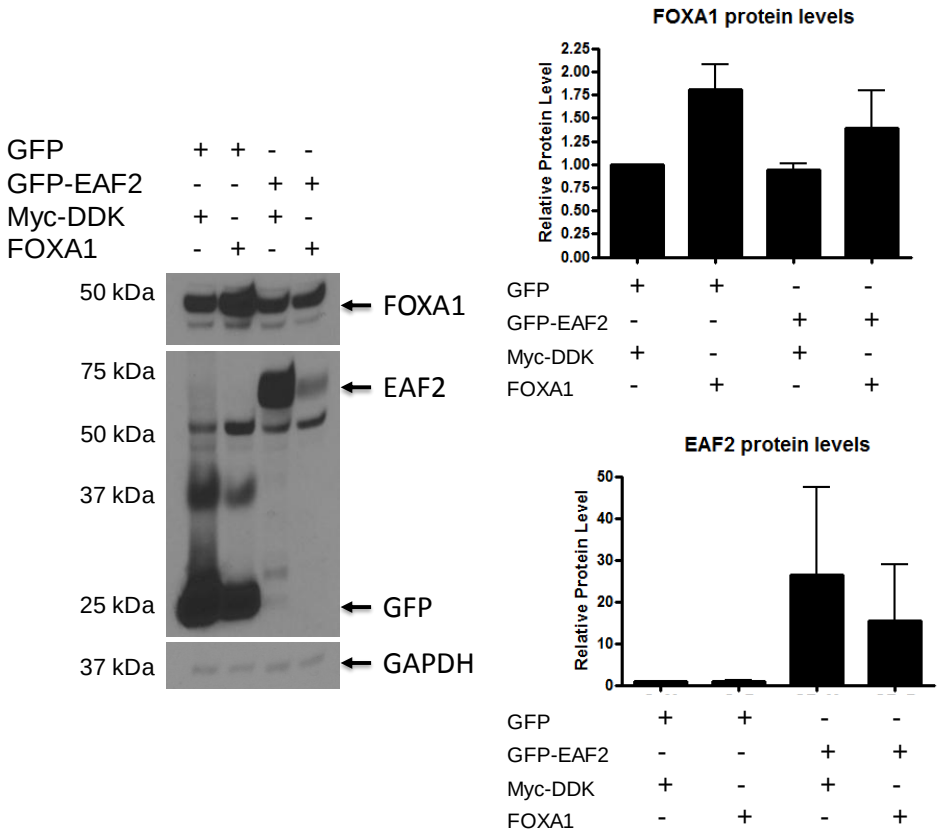

Figure 8: FOXA1 and EAF2 protein levels are reduced when co-expressed. Western blot of whole cell lysate from LNCaP cells co-transfected with GFP-tagged EAF2 (GFP-EAF2), GFP-tagged empty vector (GFP), untagged FOXA1 (FOXA1), or Myc-DDK-tagged empty vector (Myc-DDK) suggests EAF2 and FOXA1 protein levels are reduced when co-expressed. The left panel is a blot that is representative of the 3 replications and was blotted with anti-FOXA1 antibody (top blot), anti-GFP antibody (middle blot), or anti-GAPDH antibody (bottom blot). The upper right panel is a graphical summary of the densitometric analysis of FOXA1 expression normalized to GAPDH used as a loading control. The lower right panel is a graphical summary of the densitometric analysis of EAF2 expression normalized to GAPDH used as a loading control. Bars represent the means of 3 independent experiments ± SEM.

Figure 9

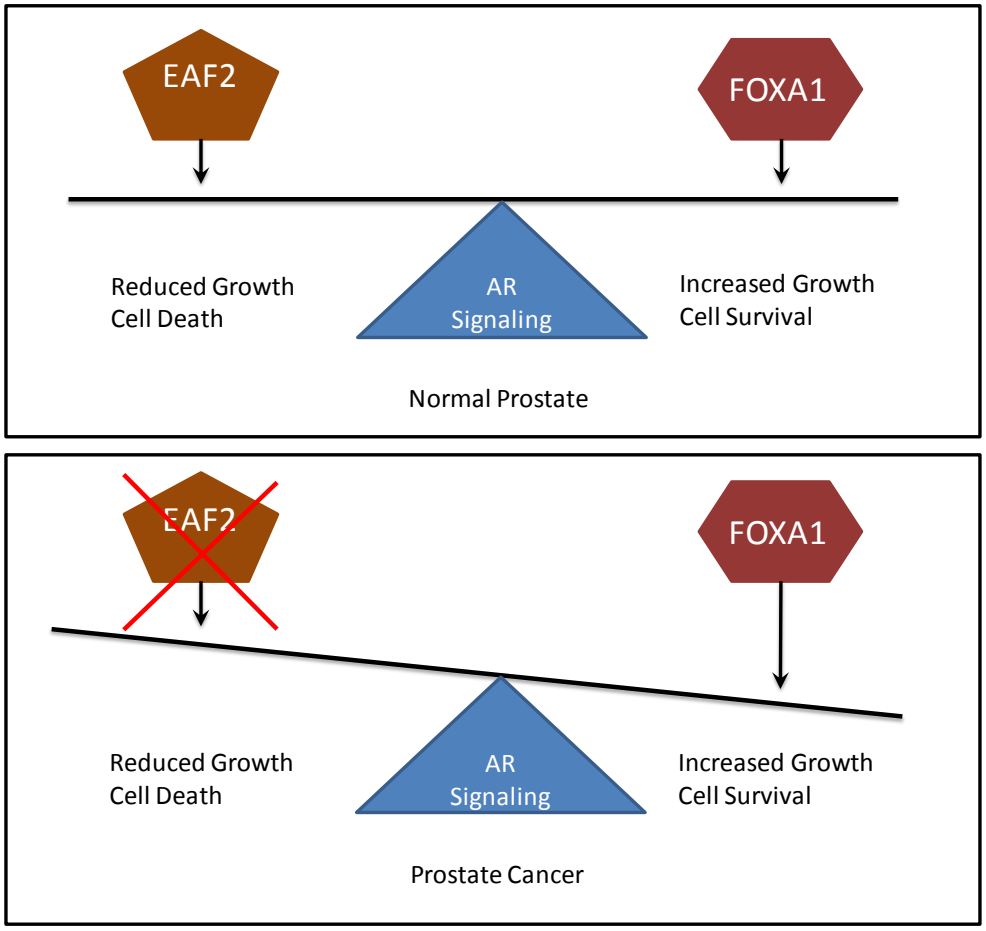

Figure 9: Hypothesized model of the interaction of FOXA1 and EAF2 modulating androgen receptor signaling. In the normal prostate epithelial cells, FOXA1 and EAF2 are both expressed. EAF2 suppresses growth and promotes cell death while FOXA1 promotes cell growth and suppresses cell death. The expression of both results in an intermediary phenotype and prostate homeostasis. In prostate cancer, EAF2 expression is reduced in early stage cancer and absent in advanced prostate cancer. Loss of EAF2 upsets the balance between increased proliferation signals and decreased proliferation signals, leading to increased cell growth and survival.

Figure 10

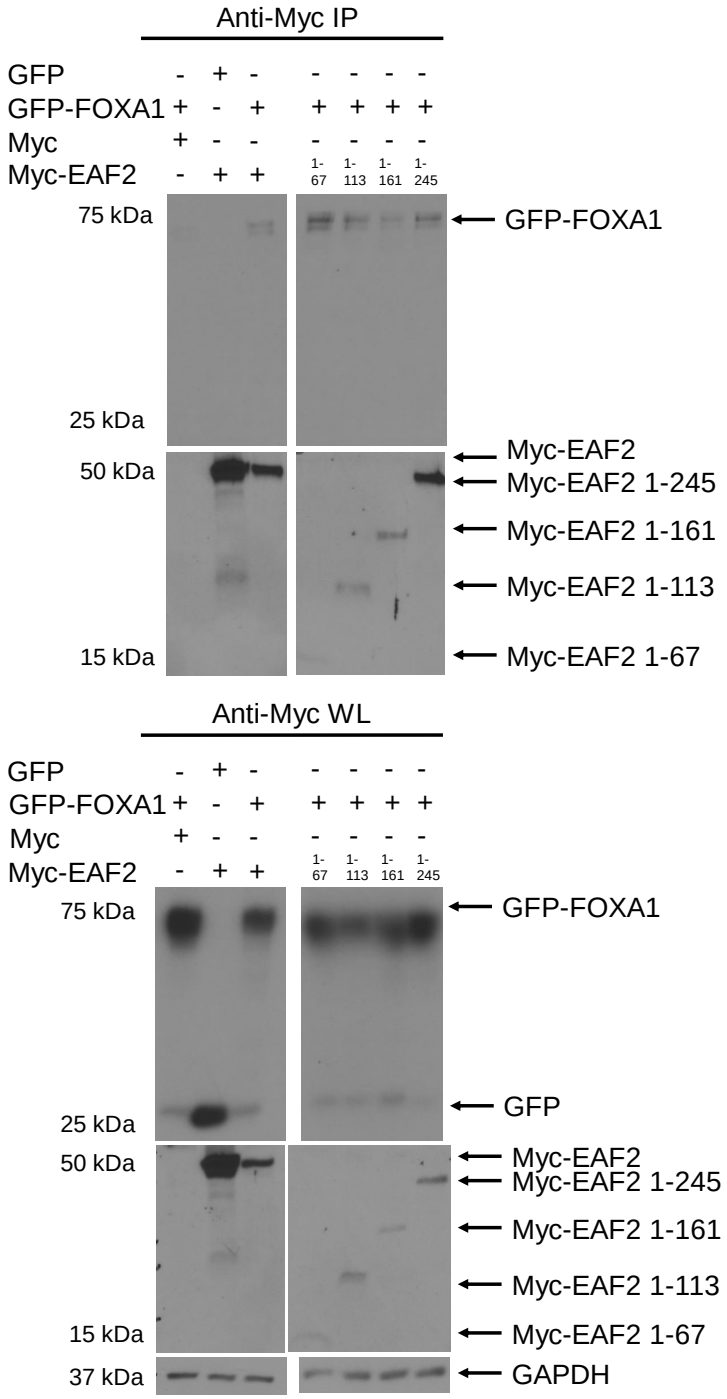

Figure 10: FOXA1 associates with the NH2-terminus of EAF2. GFP-tagged FOXA1 (GFP-FOXA1) was co-transfected with full length Myc-tagged EAF2 (Myc-EAF2), Myc-tagged EAF2 COOH-terminal deletion mutants (1-67, 1-113, 1-161, and 1-245) or Myc-tagged empty vector (Myc) into PC3 cells. Myc-EAF2 was co-transfected with the GFP-tagged empty vector (GFP) as a negative control. Cell lysates were

immunoprecipitated with anti-Myc antibody-conjugated agarose beads and blots were probed with anti-GFP antibody (1st and 3rd blot), anti-Myc antibody (2nd and 4th blot) or anti-GAPDH antibody (5th blot).

Figure 11

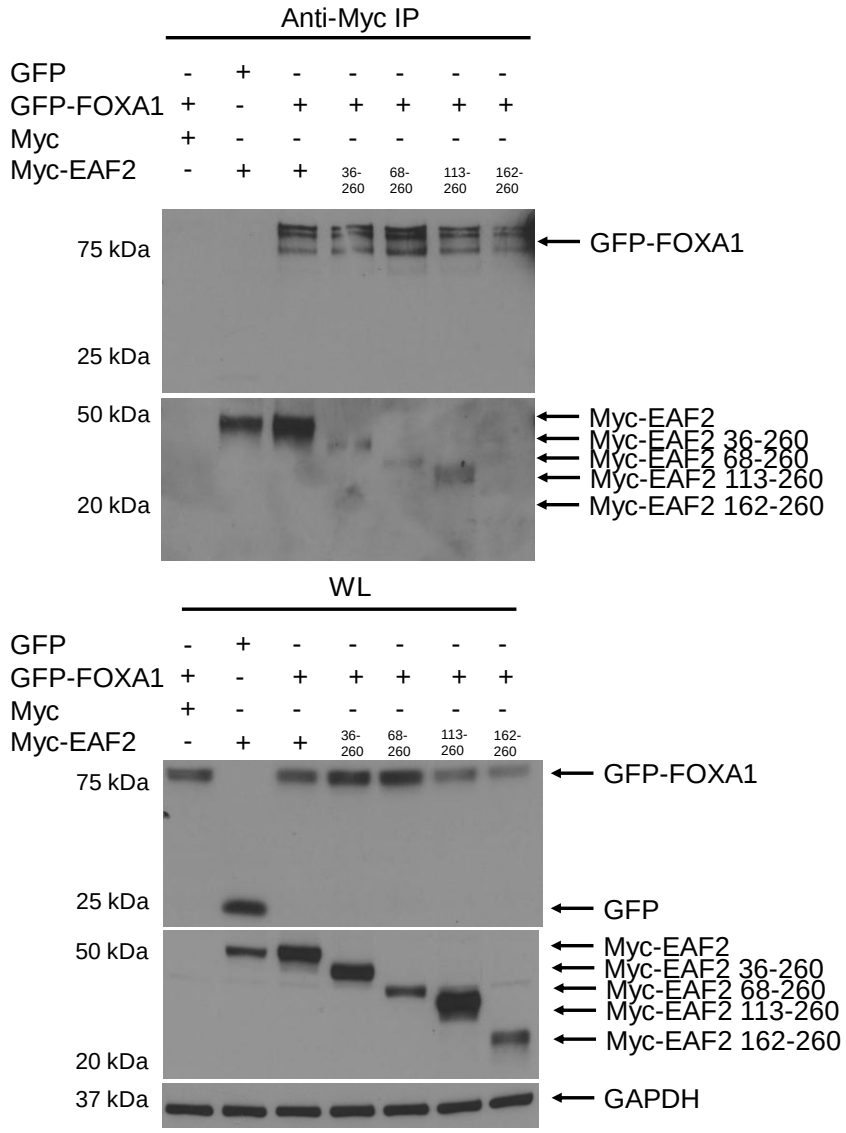

Figure 11: FOXA1 associates with the COOH-terminus of EAF2. GFP-tagged FOXA1 (GFP-FOXA1) was co-transfected with full length Myc-tagged EAF2 (Myc-EAF2), Myc-tagged EAF2 NH2-terminal deletion mutants (36-260, 68-260, 113-260, and 162-260) or Myc-tagged empty vector (Myc) into PC3 cells. Myc-EAF2 was co-transfected with the GFP-tagged empty vector (GFP) as a negative control. Cell lysates were immunoprecipitated with anti-Myc antibody-conjugated agarose beads and blots were probed with anti-GFP antibody (1st and 3rd blot), anti-Myc antibody (2nd and 4th blot) or anti-GAPDH antibody (5th blot).

Figure 12

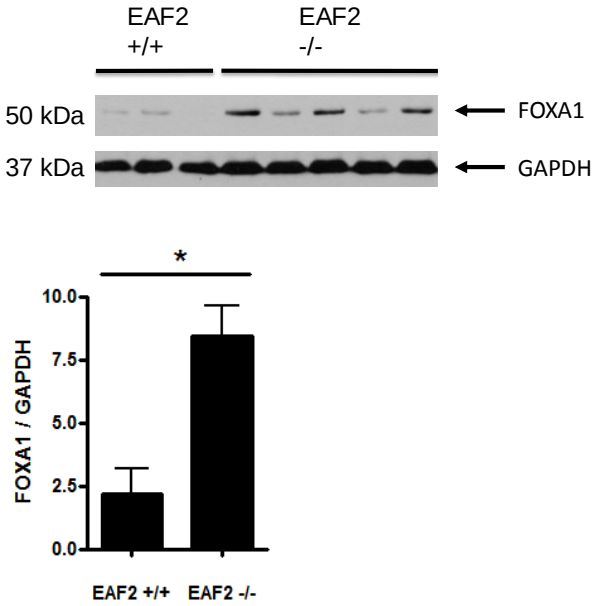

Figure 12: FOXA1 protein levels are elevated in the prostates of EAF2 knockout mice. Western blot of murine prostate tissue reveals FOXA1 is elevated when EAF2 is knocked out. Protein lysates harvested from the anterior prostates of 3 wild type (EAF2 +/+) and 5 Eaf2 knockout (EAF2 -/-) mice 24 hours after the mice were exposed to 8 gray of whole-body γ-radiation were blotted with anti-FOXA1 antibody (upper blot) or GAPDH antibody (lower blot). The lower panel is a graphical summary of the densitometric analysis of XA1 expression normalized to GAPDH used as a loading control. Bars represent the mean of the different samples ± SEM. A *t*-test was performed to determine significance. *=p≤0.05

Figure 13

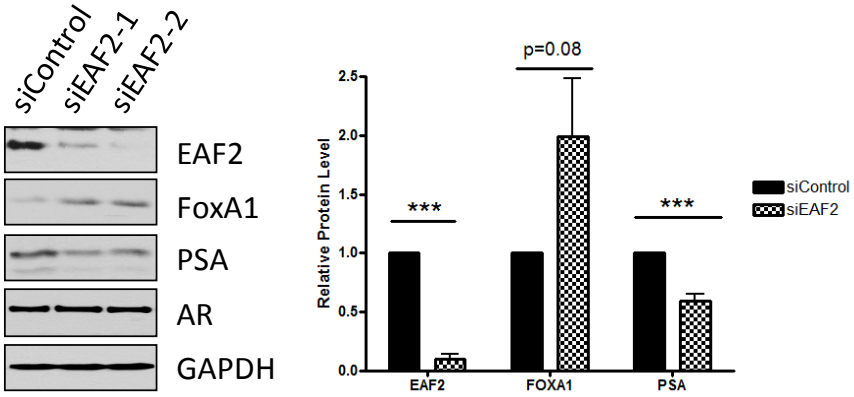

Figure 13: FOXA1 protein levels are elevated and PSA protein levels are reduced in EAF2 knockdown LNCaP cells. Western blot of EAF2 knockdown LNCaP cells reveals loss of EAF2 increases FOXA1 and decreases PSA protein levels after treatment with control siRNA (siControl) and EAF2 siRNA (siEAF2-1 and si-EAF2-2). The left panel is a blot that is representative of the 3 replications and was blotted with anti-EAF2 antibody (1st blot), anti-FOXA1 antibody (2nd blot), anti-PSA antibody (3rd blot), anti-AR antibody (4th blot) or anti-GAPDH antibody (5th blot). The right panel is a graphical summary of the densitometric analysis of EAF2, FOXA1, and PSA expression normalized to GAPDH used as a loading control. Bars represent the mean of the different samples ± SEM. A t-test was performed to determine significance. ***=p≤0.001.

Figure 14

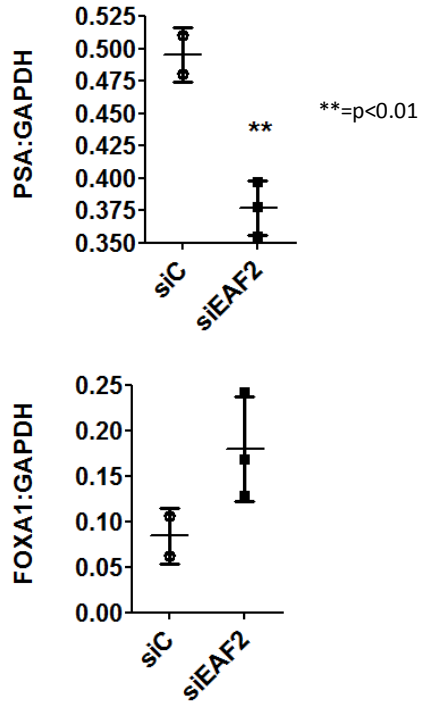

Figure 14: PSA mRNA levels are reduced in EAF2 knockdown LNCaP cells. LNCaP cells were treated with EAF2 siRNA (siEAF2) or control siRNA (siC). The cells were then subjected to reverse transcription and

quantitative real-time PCR for PSA mRNA levels (top) and FOXA1 mRNA levels (bottom). Data represents the mean ± SEM of 2 siC samples and 3 siEAF2 samples. A t-test was performed to determine significance. **= $p \leq 0.01$.

Figure 15

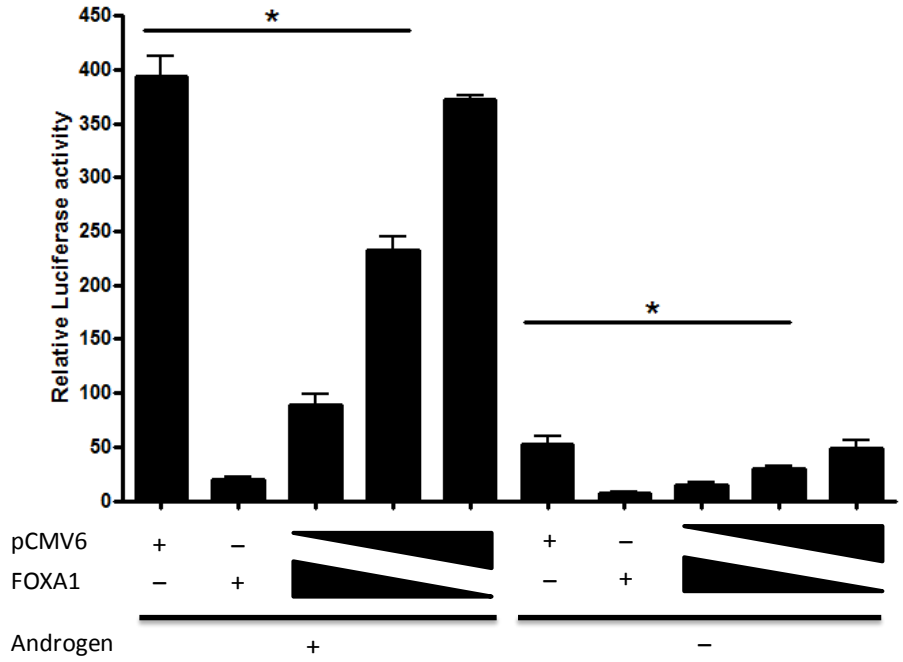

Figure 15: FOXA1 represses PSA promoter activity. C4-2 cells were transfected with 250 ng of PSA-promoter driven luciferase, 25 ng of CMV-promoter driven renilla and 250 ng of FOXA1-untagged plasmid or pCMV6 empty vector alone or with 1:1, 1:5, 1:15, and 1:25 parts FOXA1 plasmid to pCMV6 plasmid, totaling 250 ng of DNA. Cells were lysed 24 hours after treatment with charcoal-striped RPMI+ media with (+ androgen) or without (-androgen) 1 nM supplemental R1881 administered 16 hours before lysis. Luciferase expression was calculated relative to renilla expression. Significance determined by t-test. *=$p \leq 0.05$

Figure 16

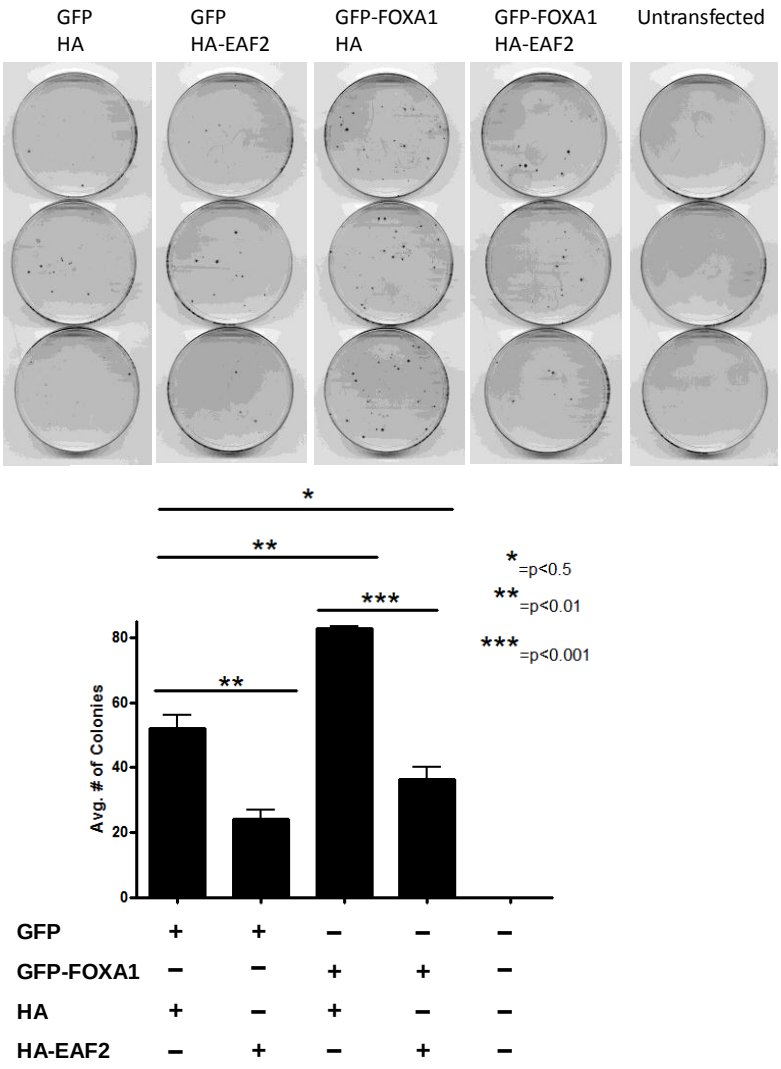

Figure 16: Co-expression of FOXA1 and EAF2 results in an intermediary growth phenotype. Surviving LNCaP cells co-transfected with HA-EAF2 and/or GFP-FOXA1 at 3 weeks stained with crystal violet (top image). Empty vector controls GFP and HA. Data depicted as mean ± SEM. Significance determined by t-test. *=p≤0.05, **=p≤0.01, ***=p≤0.001.

Figure 17

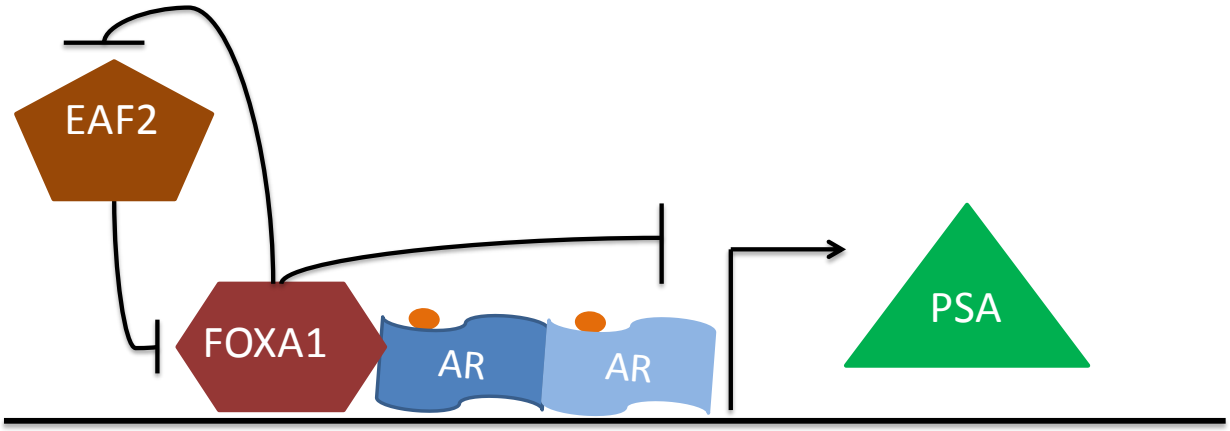

Figure 17: FOXA1 and EAF2 form a feedback loop in androgen receptor signaling. Schematic of the proposed mechanism based on the current observations of the interaction of FOXA1 and EAF2. FOXA1 binds to the androgen receptor (AR) and to a gene promoter and inhibits transcription of androgen-responsive genes like PSA. EAF2 inhibits FOXA1-mediated repression of androgen-receptor transcription and reduces FOXA1 protein levels and FOXA1 reduces EAF2 protein levels and inhibits EAF2.